Substrate Specificity of Pyridoxine Dehydrogenase from Yeast, and the Synthesis and Biological Activities of 5-Vinyl and 5-Ethynyl Analogs of Pyridoxol†

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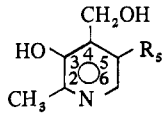
A number of 5-modified analogs of pyridoxol were found to be substrates of pyridoxine dehydrogenase. The enzyme, isolated from bakers' yeast by an improved method using DEAE-Sephadex chromatography, can readily accommodate an unbranched side chain, either saturated or unsaturated. Pyridoxol analogs were prepared with 5-vinyl by the Wittig reaction, and with 5-ethynyl by bromination and dehydrobromination of the 5-vinyl derivative. Related compounds prepared include the 5-ethyl and β-bromovinyl analogs of pyridoxol, and the *N*-oxide of the vinyl analog. The 5-vinyl analog was found to be a better inhibitor of pyridoxal phosphokinase than was the saturated analog. The significances of enzymatic findings are discussed in relation to the inhibitory activity of these compounds against *Saccharomyces carlsbergensis* and mouse mammary adenocarcinoma cells *in vitro*. Although 4-vinylpyrodoxal and some of its derivatives are highly potent B_6 antagonists, 5 isomers had only marginal activity.

5-Homologs of pyridoxol [**1**, $R_5 = (CH_2)_nOH$; n = 2-4] are potent competitive antagonists of vitamin B_6 in *Saccharomyces carlsbergensis* but, in contrast to other B_6 antagonists, such as 4-deoxypyridoxol (4-DOP), have no effect



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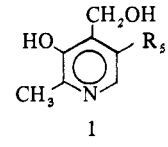
on mice or on mammalian cells grown in tissue culture.[1a,‡,3] Conversion of the 4-hydroxymethyl group to methyl, formyl, or carboxyl reduces or abolishes the inhibitory activity of these compounds.[1a] 5-Homologs are ineffective as inhibitors of pyridoxal phosphokinase[1a] and pyridoxine-P oxidase.[4]

In an attempt to determine the basis of the selectivity of 5-homopyridoxols, we studied the interaction of these analogs with pyridoxine dehydrogenase from yeast. This enzyme, known to occur only in microorganisms, catalyzes the reversible interconversion of the 4-hydroxymethyl group in pyridoxol and the 4-formyl group in pyridoxal. Pyridoxine dehydrogenase was isolated from bakers' yeast by an improved method using DEAE-Sephadex chromatography instead of the fractionation used by Morino and Sakamoto.[5]

Homopyridoxols (**1b-d**) were found to be good substrates of pyridoxine hydrogenase, as shown by the K_m values (Table I), which are comparable to those found for pyridoxol (**1a**).§ Other analogs of pyridoxol modified in the 5 position were also found to be good substrates. The ratios of analog aldehyde formed to pyridoxal formed under the conditions of maximal velocity were also determined (Table I), since they give some indication of the substrate activity whenever the K_m value is too low.

Table I. K_m Values and Substrate Activities of Pyridoxol Analogs



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No.	Substrate	K_m ($\times 10^{-3}$ *M*)[a]	Ratio of aldehyde formed to pyridoxal formed[b]
1a	Pyridoxol (5-CH_2OH)	14.3	
1b	5-CH_2CH_2OH[c]	45.5[d]	1.51[c]
1c	5-$(CH_2)_3OH$[c]	13.7	0.835
1d	5-$(CH_2)_4OH$[c]	24.4	0.755
1e	5-CH_2SH[e]	*f*	0.51
1f	5-CH_3[c]	14.3	1.45
1g	5-CH_2CH_3	1.41	1.27
1h	5-$CH{=}CH_2$	2.13	2.20
1i	5-$C{\equiv}CH$	250	1.08
1j	5-$CH(CH_3)OH$[c]	*f*	0.110
1k	5-$C(CH_3)_2OH$[c]	*f*	0.012

[a] All compounds were tested in the range from 2.08×10^{-4} to 2.08×10^{-3} *M*. [b] Determined from the samples containing the maximum substrate concentration in each case. [c] Synthesis described in ref 3. [d] It was necessary to determine a separate standard curve for this compound because of its slower reactivity with phenylhydrazine as a consequence of the formation of a hemiacetal with a six-membered ring. [e] Synthesis described by Paul and Korytnyk.[7] [f] Values were too small to plot.

The results shown in Table I indicate that the enzyme can readily accommodate a straight 5 side chain up to four carbon atoms (compare **1a-d** and **1f-i**). Branching of the 5 side chain, however, almost abolishes the substrate activity (**1j,k**). A change of 5-methyl (**1f**) to 5-ethyl (**1g**) increased the tightness of binding ten fold. A similar increase was observed in going from the hydrophilic 5-hydroxymethyl parent substance (**1a**) to the hydrophobic 5-ethyl analog (**1g**), indicating the presence of a hydrophobic region on the portion of the enzyme's active site which is complementary to the 5 position of pyridoxol analogs. The 5-vinyl analog **1h** is also a good substrate, but the 5-ethynyl compound **1i** binds poorly, although the extent of the reaction is almost equal to that for pyridoxol. The high K_m values of the homologs for pyridoxine dehydrogenase, and the relative inactivity of the oxidized products in inhibiting *S. carlsbergensis*,[1a] make this enzyme an unlikely candidate as a target for the inhibitory 5-homologs of pyridoxol.

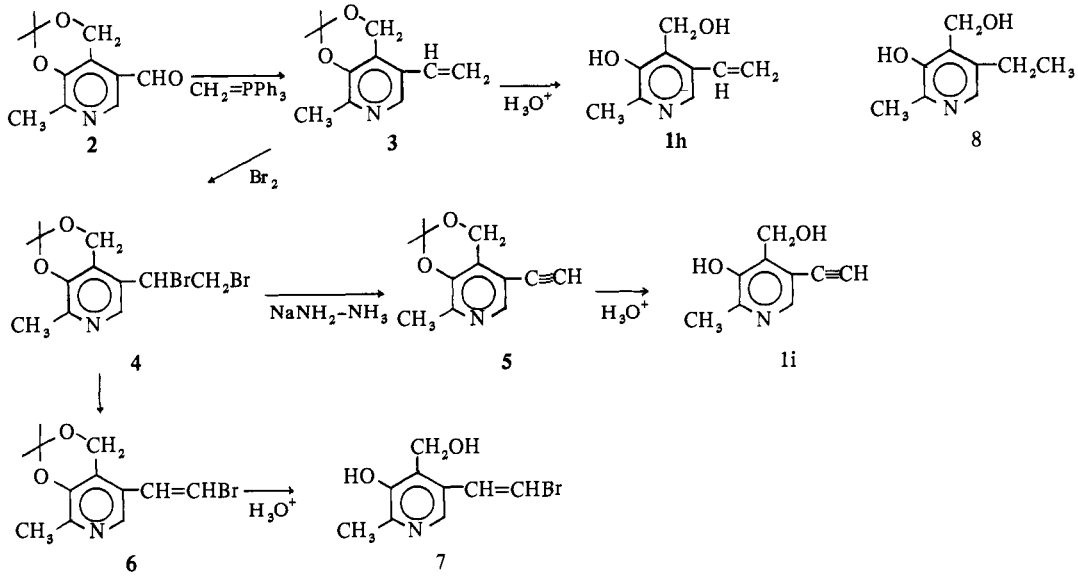
Synthesis of 5-Vinyl and 5-Ethynyl Analogs. In view of the considerable inhibitory activity of 4-vinylpyridoxal and its 5′-phosphate,[8] it is of interest to compare these com-

†Chemistry and Biology of Vitamin B_6. 29. Preceding papers in this series: ref 1a and ref 4. A brief report of part of the present study has appeared.[1b] Part of the present study was submitted by P. G. G. Potti in partial fulfillment of requirements for the Ph.D. degree.

‡For a review of the synthesis and biological activity of vitamin B_6 analogs, see ref 2.

§In this connection it is interesting to note that Melius and his coworkers[6] examined 2-homologs of pyridoxol with respect to substrate activity for pyridoxine dehydrogenase. They found that a certain amount of modification in the 2 and 6 positions (but not the 3 position) still leaves the analogs substrates of this enzyme. Thus the enzyme does not have a very high substrate specificity, as is to be expected, considering the relatively high K_m value for its natural substrate.

Scheme I



pounds with the 5-vinyl and 5-ethynyl isomers. Synthesis (Scheme I) started with isopropylideneisopyridoxal (**2**), which with methylenetriphenylphosphorane gave the blocked 5-vinyl analog **3**. The *N*-oxide of **3** was obtained by allowing **3** to react with *m*-chloroperbenzoic acid in $CHCl_3$, the vinyl group remaining intact. The target compounds **1h** and its *N*-oxide were obtained by hydrolysis of the isopropylidene group. Bromination of the 5-vinyl intermediate **3** gave the 5-(dibromoethyl) derivative **4**, which was dehydrobrominated singly to the β-bromovinyl analog **6** and doubly to the ethynyl compound **5**. Hydrolysis of the isopropylidene groups on **5** and **6** gave the two target compounds **7** and **1i**, respectively.

We determined the orientation and configuration of the monodehydrobromination products **6** and **7** by nmr spectroscopy. On the basis of the low values of the coupling constants for the α^5 and β^5 protons in **6** and **7** (J = 1.5 and 2.5 cps, respectively), these compounds must have a cis configuration for these protons. The stereochemical outcome of this elimination is not unexpected, assuming that the starting dibromide **4** has a threo configuration.

Biological Properties. The newly synthesized compounds have been found to be not only substrates of pyridoxine dehydrogenase, but also inhibitors of pyridoxal phosphokinase.# 5-Vinylpyridoxol (**1h**) inhibited the enzyme more ($K_I = 6.2 \times 10^{-4} M$) than did the corresponding saturated compound **8** ($K_I = 1.2 \times 10^{-3} M$).

At $10^{-4} M$, the 5-vinyl analog **1h** was not inhibitory, but the 5-ethynyl analog **1i** and the 5-bromovinyl compound **7** exhibited slight inhibition of mouse mammary adenocarcinoma (TA 3) cells grown in tissue culture on a synthetic medium containing $10^{-7} M$ pyridoxal.** Similarly, compounds **3**, **1h**, and **8** and the α^4,3-*O*-isopropylidene derivative of **8** inhibited the growth of *S. carlsbergensis* very poorly, even at $10^{-3} M$.††

The inactivity of the 5-vinyl and 5-ethynyl analogs of vitamin B_6 is in sharp contrast to the potency of the corresponding 4 isomers as inhibitors of TA 3 and S-180 cells in tissue culture.[8b] Whereas the 4 isomers have an unchanged 5-hydroxymethyl group and are almost certainly phosphorylated inside the cell, thus interfering with various enzymes at the phosphate level, particularly pyridoxine-P oxidase,[8b] the 5 isomers are only inhibitors of pyridoxal phosphokinase. We have found that even potent inhibitors of pyridoxal phosphokinase are not very effective as inhibitors of cells grown in tissue culture. This is true for the oximes and various hydrazones of both pyridoxal# and isopyridoxal;[10] despite the potency of such analogs as inhibitors of pyridoxal phosphokinase, they showed a weak inhibitory activity in tissue culture and also *in vivo*.** Of much greater importance as a target enzyme for inhibitory activity *in vivo* is pyridoxine-P oxidase, but it has a strict requirement for phosphorylated analogs;[4] the 5-modified derivatives obviously cannot be phosphorylated *in vivo*, and hence they are inactive.

Thus, although we have a plausible explanation for the inactivity of 5-homopyridoxols and similar compounds against mammalian cells, we have no such explanation with regard to their potent inhibitory activity against *S. carlsbergensis*. Our inability to explain their inhibitory effect on the basis of the inhibition of enzymes, such as pyridoxine dehydrogenase from yeast or pyridoxal phosphokinase, again raises the possibility suggested earlier,[3] that they interfere with the uptake of the various forms of the vitamin into the yeast cell.

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Tlc (silica gel) was used routinely as described earlier.[11] Ir spectra were determined with a Perkin-Elmer 457 spectrophotometer, and nmr spectra with a Varian A-60A instrument, using 8–15% solns in $CDCl_3$, DMSO, or D_2O; positions of peaks are expressed in cps from TMS, or from dioxane (−222 cps), as an internal std. Peaks are assigned on the basis of previous work.[12]

Purification of Pyridixone Dehydrogenase. A mixture of 10 ml (6.5 g) of dry bakers' yeast (*S. cerevisiae*) (Sigma Chemical Co., St. Louis, Mo.) and 15 ml of 0.2 *M* pH 8.0 potassium phosphate buffer was stirred until all of the yeast was suspended. This suspension was next passed ten times through a French press at 9500 lb/in.^{2} and was then centrifuged for 30 min at 70,000*g*. (The extent of the rupturing of the yeast cells was followed with a microscope.) The ppt was discarded. The supernatant (11.4 ml containing 75 mg/ml of of protein) was filtered through Miracloth, applied in 0.2 *M* pH 8.0

#From rat liver, as described by McCormick, *et al.*[9]

**Dr. M. T. Hakala, personal communications.

††Dr. A. Bloch, personal communications. Tests were performed as described in ref 3.

phosphate buffer to a DEAE-Sephadex column (2.5 × 90 cm), and eluted with the same buffer. The column required approximately 1.5 days to develop. The most active fractions were combined (total of 43 ml containing 0.43 mg/ml of protein) and used without further purification. The specific activity of this fraction was 883 nmoles of pyridoxal formed per mg of protein in 10 min at 37°. The substrate used was 25 μmoles of pyridoxol.

Substrate Activity of Pyridoxol Analogs. Assay Procedure.‡‡ For all K_m determinations, 0.2 *M* pH 9.0 Tris buffer was used because of the enhanced product yield presumably resulting from the formation of a Schiff base by the amine buffer with the aldehyde pyridoxal, shifting the reaction to the right. Approximately 29% enhancement of product yield was obtained in relation to the corresponding phosphate or bicarbonate buffer. Assays were carried out in in a darkened room.

A typical sample consisted of 0.5 ml of 0.005 *M* TPN (triphosphopyridine nucleotide), 0.5 ml of enzyme soln, pyridoxol ranging from 2.08×10^{-4} *M* to 2.08×10^{-3} *M* (final concentration), and enough Tris buffer to give a final vol of 3.0 ml. The enzyme was first allowed to associate with the TPN plus buffer with shaking for 2 min at 37°, and then the appropriate amount of substrate was added. After 10 min of shaking, 0.3 ml of 100% (W/V) TCA (trichloroacetic acid) was added, and the resulting mixture was centrifuged to remove the ppt. Then 2.0 ml of sample was mixed with 1.8 ml of H_2O. To this mixt was added 0.2 ml of phenylhydrazine soln (1.0 g of charcoal-decolorized and recrystallized phenylhydrazine hydrochloride in 50 ml of 10 *N* H_2SO_4). The tubes were placed in a 60° water bath for 20 min and then allowed to cool to room temp.

Optical density was determined at 410 nm against appropriate blanks. The K_m values, determined from Lineweaver-Burk plots, are presented in Table I. The ratios of aldehyde formed to pyridoxal formed were determined after a reaction time of 10 min, and are also given in Table I.

Syntheses. 2,2,8-Trimethyl-5-vinyl-4*H*-*m*-dioxino[4,5-*c*]pyridine (3). Triphenylmethylphosphonium bromide (5.36 g, 15 mmoles) was added under N_2 within 5 min to a stirred mixt of *n*-butyllithium (960 mg, 15 mmoles, as a 21.5 wt % solution in hexane) and a soln of 3 g of potassium *tert*-butoxide and *tert*-butyl alcohol (1:1) in 40 ml of ether. The resulting mixt was stirred at room temp for 2 hr. α^4,3-*O*-Isopropylideneisopyridoxal[10] (**2**, 21.0 g, 10 mmoles) was added within 15 min, and stirring was continued for approx 15 min. The reaction mixt was filtered, and the filtrate was extracted with saturated $NaHSO_3$ and NH_4Cl solutions, and finally with water. After drying ($MgSO_4$), the solvent was evaporated and the oily residue was distilled, yielding 1.37 g (66%) of a colorless oil, bp 88–89° at 0.4 Torr. *Anal.* ($C_{12}H_{15}NO_2$) C, H, N.

The hydrochloride of 3 was obtained by treatment with anhydrous HCl in Et_2O; it decomposes above 210°. *Anal.* ($C_{12}H_{16}NClO_2$) C, H, N.

The *N*-oxide of 3 was obtained by the dropwise addn of 3 (100 mg in 2 ml of $CHCl_3$) to *m*-chloroperbenzoic acid (250 mg in 5 ml of $CHCl_3$). The addition was completed in 15 min, and the reaction mixt was stirred for another 40 min at room temp. After the addition of more $CHCl_3$, the reaction mixt was extracted with Na_2SO_4 and $NaHCO_3$ solns and was finally washed with H_2O. After drying ($MgSO_4$), the $CHCl_3$ soln was filtered and evapd to a solid (85 mg, 81%), which was crystallized from Et_2O–petr ether: mp 157–158°; nmr ($CDCl_3$) $(CH_3)_2C$ −94, (2-CH_3) −146, (4-CH_2) −293, (5-CH=CH_2) −324 to −352 m, (5-CH=CH_2) −377 to −410 m, C_6-H −491; ir λ_{max}^{KBr} 1545 (C=C), 1120 cm^{-1} (N→O). *Anal.* ($C_{12}H_{15}NO_3$) C, H.

3-Hydroxy-4-(hydroxymethyl)-2-methyl-5-vinylpyridine Hydrochloride (1h). Compd 3 (205 mg, 1 mmole) was dissolved in 1 *N* HCl (10 ml), and was kept at 50° for 1 hr. After evaporation, the resulting crystalline material (200 mg, 99%) was recrystallized from Me_2CO–MeOH: mp 190° dec. *Anal.* ($C_9H_{12}NClO_3$) C, H, N.

The *N*-oxide of 1h was obtained by hydrolysis (heating with 1 *N* HCl on a steam bath for 1 hr) of the *N*-oxide of **3** (see above). Evaporation *in vacuo* gave a solid, which was crystallized from EtOH–Et_2O: mp 136–139°; ir λ_{max}^{KBr} 1560 (C=C), 1100 cm^{-1} (N→O). *Anal.* ($C_9H_{12}ClNO_3$) C, H.

2,2,8-Trimethyl-5-ethyl-4*H*-*m*-dioxino[4,5-*c*]pyridine Hydrochloride. A soln of 3 (20.5 mg, 1 mmole) in MeOH (15 ml) was added to a suspension of 5% Pd/C (15 mg) in MeOH (3 ml) and was hydrogenated for 45 min at room temp and atmospheric pressure. The reaction mixt was filtered, the solvent was evaporated, and the oily residue was dissolved in Et_2O. Addition of ethereal HCl precipitated the hydrochloride, which was washed with Et_2O. The yield was 230 mg (95%): mp 210° dec, from Me_2CO–MeOH. *Anal.* ($C_{12}H_{18}NO_2Cl$) C, H, N, Cl.

3-Hydroxy-2-methyl-4-(hydroxymethyl)-5-ethylpyridine (8) Hydrochloride. The product from the preceding experiment (50 mg) was dissolved in 1 *N* HCl (4 ml) and kept at 50° for 1 hr. After evaporation *in vacuo*, it was recrystallized from Me_2CO–MeOH. The yield was 38 mg (91%): mp 138°. *Anal.* ($C_9H_{14}NClO_2$) C, H, N, Cl.

α^4,3-*O*-Isopropylidene-α^5,β^5-dibromoethylpyridoxol (4). To a stirred soln of 3 (510 mg, 2.15 mmoles) in CCl_4 (4 ml, dry), a soln of Br_2 (480 mg, 3 mmoles) in CCl_4 (1 ml, dry) was added for 15 min. After 30 min, no starting material remained (tlc). The solvents were evaporated *in vacuo*, and the oily residue was dissolved in Et_2O and dried ($MgSO_4$). Evaporation yielded an oil (840 mg, 94%), which was characterized as the hydrobromide: mp 187–188°. *Anal.* ($C_{12}H_{16}Br_3NO_2$) C, H, Br. The hydrochloride was obtained from dry ethereal HCl: mp 195–198°.

α^4,3-*O*-Isopropylidene-α^5-deoxy-α^5-methylidynepyridoxol (5). Fresh $NaNH_2$ was prepared according to the method of Vaughn, *et al.*[14] Liquid NH_3 (25 ml) was collected in a three-necked flask and cooled in a mixt of Dry Ice and Me_2CO. Then $Fe(NO_3)_2$ (2 mg) was added, followed by Na (5 mg), with magnetic stirring, moisture being excluded. After 1 min, the blue color of the soln faded and was completely discharged by the passage of dry air. Now 115 mg of Na was added in small pieces (chunks) during 10 min. After the color became gray, 3 mg of aniline was added as a catalyst. The soln of $NaNH_2$ thus obtained was cooled and vigorously stirred for 1 hr, and a soln of the dibromo compound **4** (500 mg, 1.4 mmoles) in Et_2O (anhydrous, 30 ml) was added dropwise during that time. Stirring was continued for another 2 hr. The sodium acetylide formed was hydrolyzed by the addition of concentrated NH_4OH (20 ml), followed by H_2O. Excess $NaNH_2$ and NH_4OH were decomposed by careful addition of dilute HCl while cooling with a mixt of Dry Ice–EtOH. Extraction with Et_2O (3x), washing the extract with H_2O, and drying yielded crystalline material (220 mg, 78%), which was purified by sublimation with a water pump: mp 70°; nmr ($CDCl_3$) $(CH_3)_2C$ −93, (2-CH_3) −146, (4-CH_2) −292, 5-C≡CH −203; ir λ_{max}^{KBr} 3390 cm^{-1} (C≡CH stretching). *Anal.* ($C_{12}H_{13}NO_2$) C, H, N. The hydrochloride was obtained from ethereal HCl: mp 150°.

α^5-Deoxy-α^5-methylidynepyridoxol (1i) Hydrochloride. The free base (5, 100 mg) was hydrolyzed with 0.3 *N* HCl on a steam bath for a few minutes. The solvent was removed *in vacuo*, and the compound was crystallized from EtOH: mp 185–186°; nmr (NaOD, 1 *M*) (2-CH_3) −155, (4-CH_2) −302, C≡CH −243, (C_6-H) −494; ir λ_{max}^{KBr} 3260 (C≡CH stretching), 3050 cm^{-1} (C≡C stretching). *Anal.* ($C_9H_{10}ClNO_2$) C, H, N.

α^4,3-*O*-Isopropylidene-α^5-deoxy-α^5-(bromomethylene)pyridoxol (6). The dibromo derivative (4, 182 mg) was added to a soln of KOH (87 mg) in EtOH (5 ml), and the mixt was heated at 40–45° for 5 hr. The resulting soln was poured into H_2O, the mixt was extracted with Et_2O, and the Et_2O soln was washed with water and dried ($MgSO_4$). Evaporation of Et_2O yielded 125 mg of an oily residue, which was converted into a hydrochloride with HCl in Et_2O. The hydrochloride was crystallized from acetone: mp 162–164°; nmr ($CDCl_3$) [$(CH_3)_2C$] −92, (2-CH_3) −155, (4-CH_2) −293, (β^5-H) −350 (d, J = 1.5 cps), α^5 H-354 (d, J = 1.5 cps), (C_6-H) −481; ir λ_{max}^{KBr} 1545 cm^{-1} (C=C stretching), absence of strong band at 965 cm^{-1} indicates cis configuration of the Br. *Anal.* ($C_{12}H_{15}BrClNO_2$) C, H, halogens.

α^5-Deoxy-α^5-(bromomethylene)pyridoxol (7) Hydrochloride. Compd 6 (100 mg) was heated in 0.3 *N* HCl for a few minutes on a steam bath. The H_2O was evaporated *in vacuo*, and the hydrochloride was crystallized from EtOH: mp 180–181°; nmr (D_2O) (2-CH_3) −155, (4-CH_2) −299, (β^5-H) −363 (d, J = 2.5 cps), (α^5-H) −270 (d, J = 2.5 cps), (C_6-H) −491; ir λ_{max}^{KBr} 1530 cm^{-1} (C=C). *Anal.* ($C_9H_{11}BrClNO_2$) C, H, halogen.

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‡‡This assay procedure was adapted from Wada and Snell.[13]

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Absolute Configurations of the Four Warfarin Alcohols†

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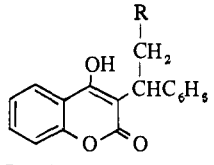
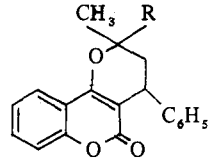
Chemical synthesis, specific stable isotope labeling, mass spectrometry, and nmr spectroscopy are utilized to establish the absolute configurations of the four stereoisomers of 3-[α-(2-hydroxypropyl)-benzyl]-4-hydroxycoumarin. The absolute configurations of the four stereoisomers of 2,3-dihydro-2-methyl-4-phenyl-4*H*-pyrano[3,2-*c*]benzopyran-5-one are also reported. Preliminary biological data indicate that in man the *"in vivo"* reduction is stereoselective and that the resultant metabolites are biologically active although not as active as warfarin itself.

In an earlier report we demonstrated that the diastereomeric alcohols (**1**), resulting from reduction of the carbonyl group in the side chain of warfarin (**2**), occur as metabolites in normal man.[2] Since phenprocoumon (**3**) and cyclocoumarol (**4**) are active as anticoagulants,[3] it appeared that minor modification of the side chain would not drastically affect this property. It seemed reasonable, therefore, to anticipate that the various isomers of **1** might have significant biological activity in their own right. In addition, the *S* forms of **2**[4] and **3**[5] are more potent than the corresponding *R* forms. For these reasons we initiated a study to establish the absolute configuration of the four possible isomers to determine their relative biological potencies as anticoagulants, and to determine the stereochemistry of the *"in vivo"* reduction of warfarin.



1a, R = $CHOHCH_3$, *R,S* or *S,R*
1b, R = $CHOHCH_3$, *R,R* or *S,S*
2, R = $COCH_3$
3, R = CH_3

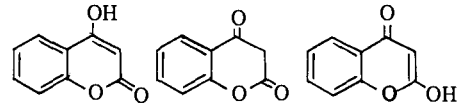


4, R = OCH_3
5a, R = H, *R,S* or *S,R*
5b, R = H, *R,R* or *S,S*

Diastereomers **1a** and **1b** can readily be obtained by reducing sodium warfarin with $NaBH_4$ followed by separation utilizing a combination of elution chromatography and fractional recrystallization. These isomers can also be separated utilizing preparative tlc. The faster moving isomer (**1a**) was characterized as described below; the slower moving material was designated **1b**.

In the initial development of tlc systems for the separation of the diastereomeric alchols it was noticed that with time a third spot appeared with higher R_f which seemed to originate from the breakdown of **1b**. This compound was isolated and characterized by mass spectroscopy and nmr as **5**. Subsequently, it was found that it could also be obtained by thermal or Lewis acid catalyzed cyclic dehydration of **1b**. Under similar conditions **1a** afforded a different isomer of **5**. Formation of cyclic analogs **5a** and **5b** are of particular significance, since the conformational possibilities are now limited and assignment of stereochemistry based on nmr spectroscopy is less ambiguous than similar analysis of the respective precursors **1a** and **1b**. If the relative stereochemistry of the isomers of **5** can be determined and the mechanism of their formation from the isomers of **1** elucidated, then it follows that the stereochemistry of the flexible isomers of **1** will also be known.

It had been pointed out by earlier investigators[6] that 4-hydroxycoumarin (**6**) can exist in two other tautomeric forms, the diketo structure (**7**) and the chromone structure (**8**). The contribution of **7** is considered minimal because of the lack of stabilization by conjugation. The existence of the tautomeric forms **6** and **8** has been demonstrated by Arndt[6a] who obtained a mixture of 4-methoxycoumarin and 3-methoxychromone on treatment of 4-hydroxycoumarin with diazomethane.



6 **7** **8**

The determination of chromone or coumarin structures for **5a** and **5b** is important to this work, since two structurally different isomers could result upon dehydration of the alcohols. Neither nmr nor mass spectroscopy can *a*

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